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Short communication

Incremental generation of an EST set for the analysis of scrapie pathogenesis

A.G. Gossner*, J. Hopkins

The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK

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ABSTRACT

As part of a functional genomic study of scrapie pathogenesis we have generated a complex non-redundant microarray from several tissues by the selection of unique clones and the incremental augmentation of the expressed sequence tag sets. The base set of about 15,000 expressed sequence tags had minimum redundancy and originated from bovine brain. The aim was to augment this set with clones from scrapie-infected brain and spleen and uninfected spleen with minimum redundancy. This was achieved by macroarray hybridization to identify unique sequences in the ovine library. Quality sequence of 4052 unique sheep clones were obtained from the sequencing of only 5000 which have been submitted to the dbEST database at GenBank.

1. Introduction

Scrapie is a naturally occurring infectious disease of sheep and is the prototype transmissible spongiform encephalopathy (TSE), a group of diseases that also includes the human Creutzfeldt–Jakob diseases (CJD), bovine spongiform encephalopathy (BSE) and chronic wasting disease (CWD). The TSEs are characterized by the alteration of a normal membrane protein PrP; from its mostly α-helical conformation (PrP^{C}), to a protease resistant fibrillar form (PrP^{SC}), largely consisting of β-sheet structure (Riek et al., 1997). The disease-associated conversion of PrP^{C} to PrP^{SC} is associated with TSE infectivity (Prusiner, 1982; Scott et al., 1997) although the link is not absolute (Barron et al., 2007). The main target organ for scrapie is the brain, but studies in mice also indicate that follicular dendritic cells of the spleen are necessary for prion propagation in the lymphoreticular system (Brown et al., 1999). Scrapie pathogenesis is still not completely understood and the use of functional genomics would allow for the molecular basis of disease development to be better understood.

The use of microarrays permits simultaneous measurement of the expression of thousands of transcripts each of which requires sequence data for annotation. To provide a relevant resource for this study requires expressed sequence tags (ESTs) from all target tissues. However, the generation of separate annotated EST sets from each tissue is uneconomical and the simple addition of EST sets produces excessive redundancy. For this study we exploited a partially annotated bovine brain EST set, with a low level of redundancy, and augmented it incrementally with ESTs from scrapie-infected brain and spleen, and uninfected spleen.

2. Materials and methods

2.1. Preparation of mRNA

Total RNA was extracted, using the RiboPure™ Kit (Ambion, Warrington, UK), from medulla (obex) and spleen from two Cheviot sheep showing clinical signs of natural scrapie, and from the spleen of three normal non-scrapie sheep. RNA quality and integrity was assessed using a RNA LabChip on the Agilent 2100 bioanalyser (Agilent, Stockport, UK). The isolated total RNA was pooled and then used to prepare poly-A mRNA with Poly (A) Purist™ MAG Kit as described by the manufacture (Ambion).

* Corresponding author. Tel.: +44 131 650 7943; fax: +44 131 650 6511.
E-mail address: A.Gossner@ed.ac.uk (A.G. Gossner).

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2.2. Construction of cDNA libraries

A directionally cloned cDNA library was constructed using Creator™ SMART™ Kit (Clontech, Saint-Germain-en-Laye, France) cloned into Sfi I-digested pDNR-LIB vector. Recombinant pDNR-LIB vector were used to transform ElectroMax™ DH5α-E™ competent cells by electroporation (Invitrogen Ltd., Paisley, UK). Another directional cDNA library was constructed using the SUPERSCRIPT™ Plasmid System and the Not I-Sal I-digested pSPORT 1 vector (Invitrogen). Recombinant pSPORT 1 was used to transform UltraMax™ DH5α-FT (Invitrogen) for the production of single-stranded DNA (ssDNA) using the helper phage M13KO7. Both libraries were amplified according to the manufacturers' protocols before normalization.

2.3. Production of single-stranded library DNA

The amplified double-stranded pSPORT 1 library was grown under ampicillin selection and converted to single-stranded circles *in vivo* after super infection with M13K07 as described by (Vieira and Messing, 1987).

2.4. PCR amplification of biotin-labelled library DNA

The pDNR-LIB library was used as a template for amplification of biotin-labelled cDNA inserts using the Expand High Fidelity^{PLUS} PCR System (Roche Diagnostics, Burgess Hill, UK) with the pDNR-LIB and 5′-biotinylated HBX primers (Table 1). Conditions of amplification: 94 °C denaturation for 7 min; 30 cycles at 94 °C for 1 min, 50 °C for 2 min, 72 °C for 3 min and a final 7 min at 72 °C. The PCR products were purified by High Pure PCR Purification Kit (Roche) and precipitated with sodium acetate and ethanol before resuspending in 5 μl of 3′ blocking oligonucleotide (1.5 μg/μl) (Table 1).

2.5. Normalization of cDNA library

Normalization of the pSPORT 1 library was adapted from Bonaldo et al. (1996) and Soares and Bonaldo (1998). A 50 ng aliquot of ssDNA was mixed with 2 μl biotin-PCR product from the pDNR-LIB library and 15 μl deionised formamide. To block hybridization via the common vector and poly(A) tail sequences, 5′ and 3′ blocking oligonucleotides were designed (Table 1) and included in the hybridization reaction. After the addition of 5 μl of 5′ blocking oligonucleotides (1.5 μg/μl) and 5 μl of 3′ blocking oligonucleotides (2 μg/μl) the hybridization mixture was heated to 80 °C for 3 min, followed by the addition of 1.5 μl of 10× Buffer A (1.2 M NaCl, 0.1 M Tris, pH 8.0 and 50 mM EDTA) and 0.5 μl of nuclease-free water. The reaction mixture was hybridized at 30 °C for 24 h after which it was gently mixed with 300 μl of ice-cold 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate) before incubation with 0.6 ml Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs), washed and prepared as per the manufacturer's instructions (Promega, Southampton, UK). The hybridization mixture and SA-PMPs were incubated at room temperature for 10 min, inverted every 1–2 min before the SA-PMPs were captured using a magnetic stand. The supernatant containing the unhybridized single-stranded circles was removed, precipitated at −80 °C with 5 μl yeast tRNA (2 μg/μl) by 150 μl of 7.5 M sodium acetate and 600 μl of ethanol. The ssDNA was pelleted by centrifugation, washed with ethanol and air-dried before resuspension in 11 μl of nuclease-free water.

The ssDNA was converted into dsDNA using Sequenase™ Version 2.0 DNA polymerase (USB, Staufen, Germany). The ssDNA was incubated at 65 °C for 5 min with 4 μl of 5× Sequenase buffer and 1 μl SP6 primer (1 μg/μl) and then 3 min at 37 °C, followed by the addition of 1 μl of DTT (100 mM), 2 μl dNTPs (100 mM each) and 1 μl Sequenase before continuing the incubation at 37 °C for 30 min. The reaction was terminated with 80 μl of 1 mM EDTA in 10 mM Tris (pH 8), followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and then chloroform:isoamyl alcohol (24:1). The DNA was precipitated at −80 °C with 150 μl of 7 M ammonium acetate, 600 μl of ethanol and 5 μl yeast tRNA (10 mg/ml). The precipitated library DNA was pelleted by centrifugation and resuspended in 4 μl of nuclease-free water.

A 2 μl aliquot of library DNA was used to transform ElectroMax™ DH10B™ T1 phage resistant competent cells (Invitrogen) by electroporation and the cells propagated under ampicillin selection to generate the normalized sheep cDNA library.

Table 1
Primer and blocking oligonucleotide sequences.

Primer	Sequence (5′–3′)
pDNR-LIB primer	ACGAAGTTATCAGTCGACGGTAC
SP6 primer	ATTTAGGTGACACTATA
5′-biotinylated HBX primer	Biotin-CCAACGAATGGTCTAGAA-AGC
3′ blocking oligonucleotide	GAAGTTATCAGTCGACGGTACCGGA-CATATGCCCGGGAATTCGGCCATTA-CGGCCGGG
5′ blocking oligonucleotide	TGGTCTAGAAAGCTTCTCGAGGGC-CGAGGCGGCCGACATGT$_{25}$
M13 reverse primer	GGACACACTTTAACAATAGGCGA

2.6. Macroarrays: subtractive hybridization of cDNA libraries

To supplement the previously constructed Bovine Brain Unigene Set of cDNA clones (http://www.imagenes-bio.de/products/sets_libraries/) consisting of 14,989 unique sequences with unique clones from the sheep cDNA library, subtractive hybridization was used to reveal common clones. Briefly, the normalized sheep library was plated on agar plates under ampicillin selection. Single colonies were picked from the agar plates and grown in 384 well plates prior to spotting on four nylon filters (22 cm × 22 cm), with 18,432 colonies per filter. The filters were incubated with radiolabelled transcripts prepared from the bovine brain set. After hybridization and exposure to phosphorimager plates, colonies from the sheep cDNA library were selected for DNA sequencing. These clones were judged not to be represented in the bovine brain set by failure of any radiolabelled probe to hybridize to the sheep library colonies.

2.7. DNA sequencing

Prior to sequencing, the clones were screened by PCR and agarose gel electrophoresis to ensure the presence of an insert. From the sheep cDNA library, 5000 clones not represented in the bovine set were selected for sequencing (MWG, Ebersberg, Germany). Single-pass sequencing was performed from the 5′end of the directionally cloned insert using M13 reverse primer (Table 1).

2.8. Sequence analysis

The chromatogram files from the sequencing were processed for base calling by Phred and the raw sequence files were further processed using Crossmatch to trim vector sequence, poly(A) tail and low quality bases from the sequences (Ewing et al., 1998; Ewing and Green, 1998). These programs were implemented within the Trace2dbest software (Parkinson et al., 2004) and used to process the sequence trace files into quality checked sequences prior to submission in the dbEST database for ESTs at GenBank (Boguski et al., 1993).

The quality EST sequences were further processed within Partigene to identify redundancy and presence of novel sequences by clustering, using the Clobb software program (Parkinson et al., 2002). The non-redundant sets of clustered sequences were assembled into consensus sequences using Phrap (Ewing et al., 1998; Ewing and Green, 1998). Preliminary gene annotation was performed using BLASTx (Altschul et al., 1990) with the non-redundant protein database at the National Center for Biotechnology Information (NCBI) to provide preliminary gene annotation from the consensus sequences (Table S1).

Functional annotation of the sheep EST sequences based on the Gene Ontology (Ashburner et al., 2000) vocabulary was performed using the Blast2GO software suite (Conesa et al., 2005). The GO annotation was refined using the generic GO Slim mapping and the reduced high-level GO term annotation is shown in Fig. 2.

3. Results and discussion

From the 73,728 sheep clones arrayed on the filters 5000 were selected for single-pass sequencing. After processing 4052 quality EST sequences (Table S1) were submitted to dbEST database, with an average length of 732 base pairs per sequence.

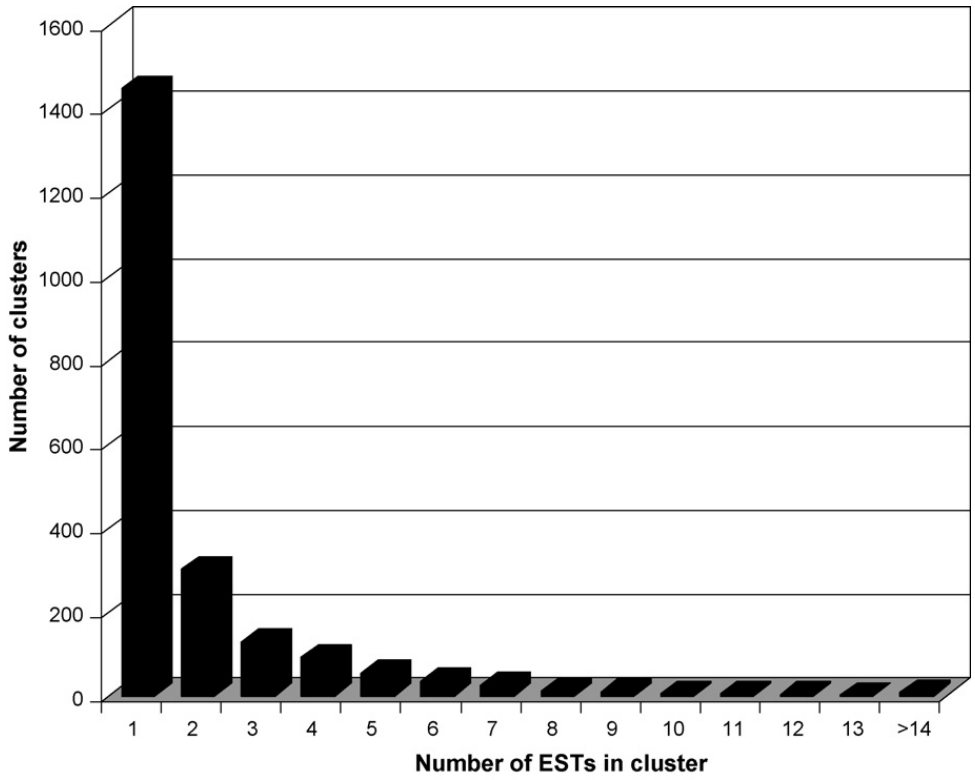

Fig. 1. Clustering results of ovine quality EST sequences using programs within Partigene, illustrating the overall sequence redundancy of the selected cDNA library. Within the library there were 1449 singletons and the majority of clusters (1880) contained one, two or three ESTs per cluster, confirming successful normalization of the library. The remaining 1244 sequences formed 159 unique clusters with 5 or more ESTs per cluster.

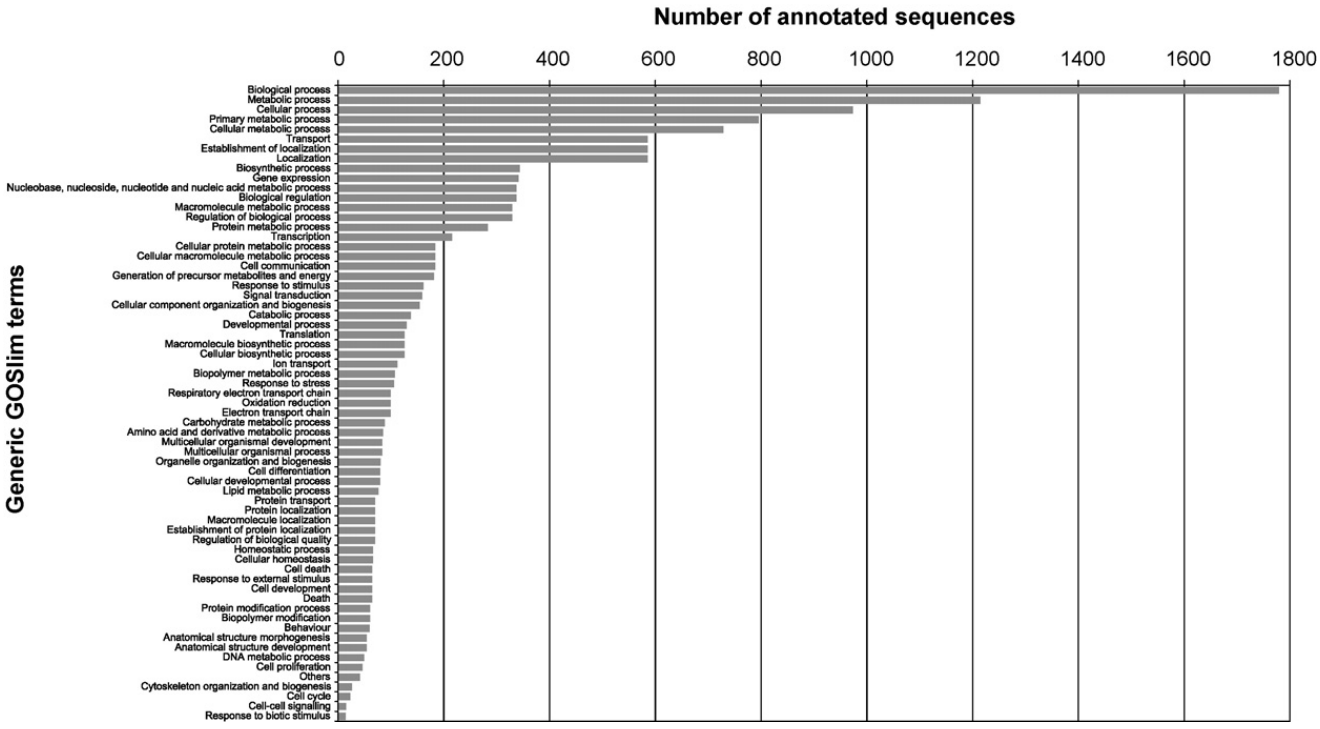

Fig. 2. Categories of biological processes identified by gene ontology (GO) mapping. Sheep EST sequences were mapped to numerous biological processes of the hierarchical GO system. These GO annotations were then condensed using the generic GOSlim mapping implemented within the Blast2GO software suite. Biological process terms containing less than 10 sequences were combined and are shown as 'Others' within the figure.

The results of clustering and assembly into consensus sequences of the sheep EST sequences are shown in Fig. 1. Of the 4052 quality ESTs, 1449 (35.8%) were singletons and the majority (1359) of the remaining sequences formed 523 clusters containing 2, 3 or 4 ESTs per cluster. Analysis of BLASTx output for ovine EST data submitted to GenBank indicated that 29% of sequences had a significant (score > 300) similarity to sequences in the GenBank non-redundant protein database.

Gene ontology based functional categorization (Fig. 2) showed the sheep EST sequences covered a wide range of biological functions.

The ovine clones selected from this library will be part of a project used to construct a ~20,000 non-redundant ruminant cDNA microarray chip and used for the analysis of mRNA transcripts in scrapie-infected sheep brains and secondary lymphoid organs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.smallrumres.2008.11.005.

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